ENHANCING BANANA DISEASE SURVEILLANCE
Case studies for BXW and BBTD

Idd Ramathani and Fen Beed
IITA-UGANDA PLANT PATHOLOGY

Background about IITA

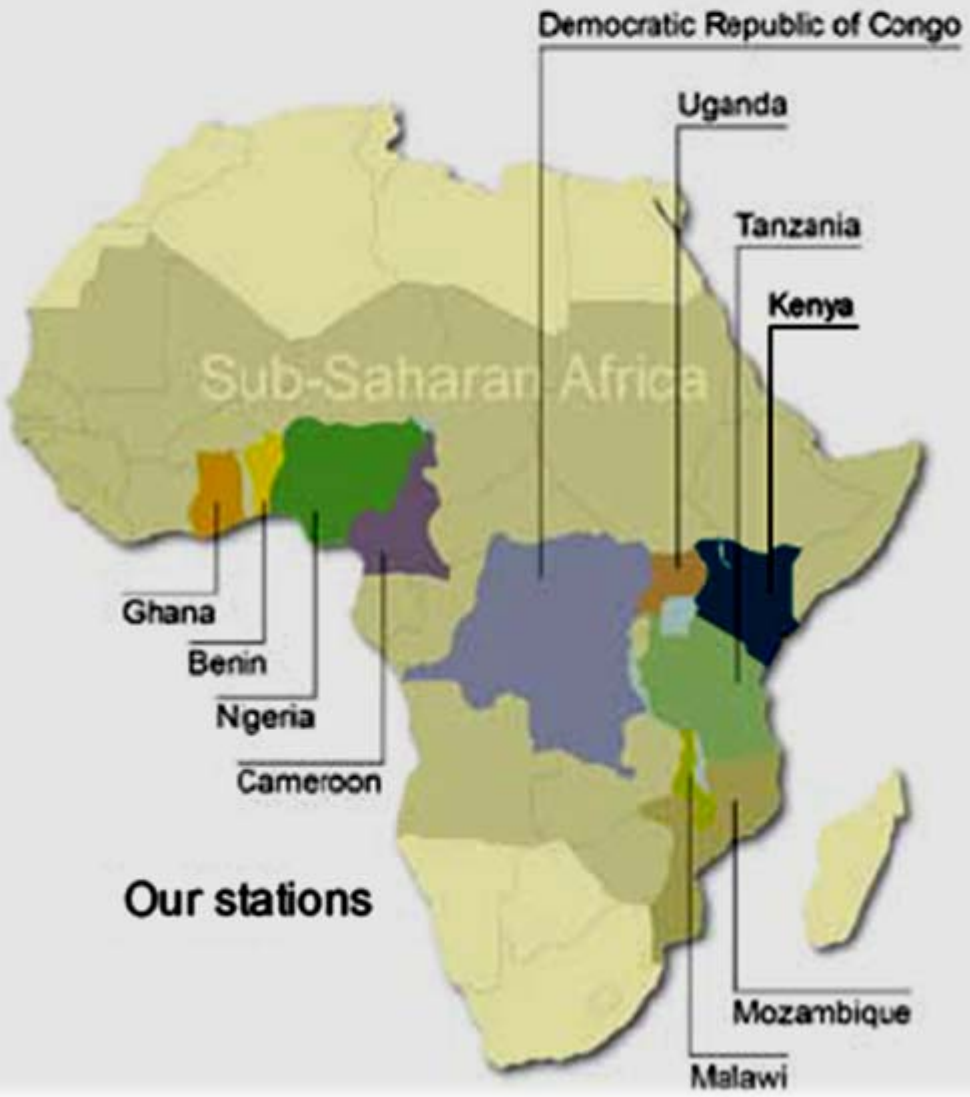
Africa has complex problems that plague agriculture and people's lives. We develop agricultural solutions with our partners to tackle hunger and poverty. Our award winning research-for-development (R4D) is based on focused, authoritative thinking anchored on the development needs of sub-Saharan Africa. We work with partners in Africa and beyond to reduce producer and consumer risks, enhance crop quality and productivity, and generate wealth from agriculture.

IITA is an international non-profit R4D organization since 1967, governed by a Board of Trustees, and supported primarily by the CGIAR (www.cgiar.org).

IITA crops

- - Global: Cowpea, Soybean, Bananas, Plantain, and Yams
  - Sub-Saharan Africa: Cassava and Maize

Where we work



Our stations

What we do.

- Agriculture and health.
- Agro-biodiversity
- Banana and Plantain
- Cereals and Legumes
- Horticulture and Tree crop
- Opportunities and threats-pests and diseases and draught
- Roots and Tubers –Programs aimed to reduce pre &post harvest losses and increase the productivity of root and tuber crops for food security






IITA-UGANDA

- Hosted under the National Research Organisation (NARO) in Uganda at two research Station
- National Biotechnology centre ,KARI, Kawanda and National Crop Research Resource Institute (NaCRRI) , Nalumonge
- http://www.banana.go.ug/

KARI,

Banana

horticulture,

Post-harvest crop losses,

soils and soil fertility

NaCRRI

-Banana research

-Coffee research

-Bean research

Cassava research

-Cereals

-Horticultural

-Potato

Banana Production in Sub-Saharan Africa

- Bananas are the fourth most important food crop in the tropical and sub-tropical zones of the world. Annual banana production in the world is estimated at 104 million tons of which less than 10% enters the commercial market, suggesting that the crop is more important as food for local consumption than for export (FAOSTAT, 2004).

- Most bananas produced in Africa are used as a staple food and a source of cash income for small-holder farmers. In some of the African countries such as Uganda, the daily consumption of banana may exceed 1.6 kg per person (FAOSTAT, 2001), highest in the world. East Africa is the largest banana producing and consuming region in Africa with Uganda being the world's second leading producer with the total production of about 10.5 million tons (FAOSTAT, 2004).

A few of the Cultivars grown in Uganda

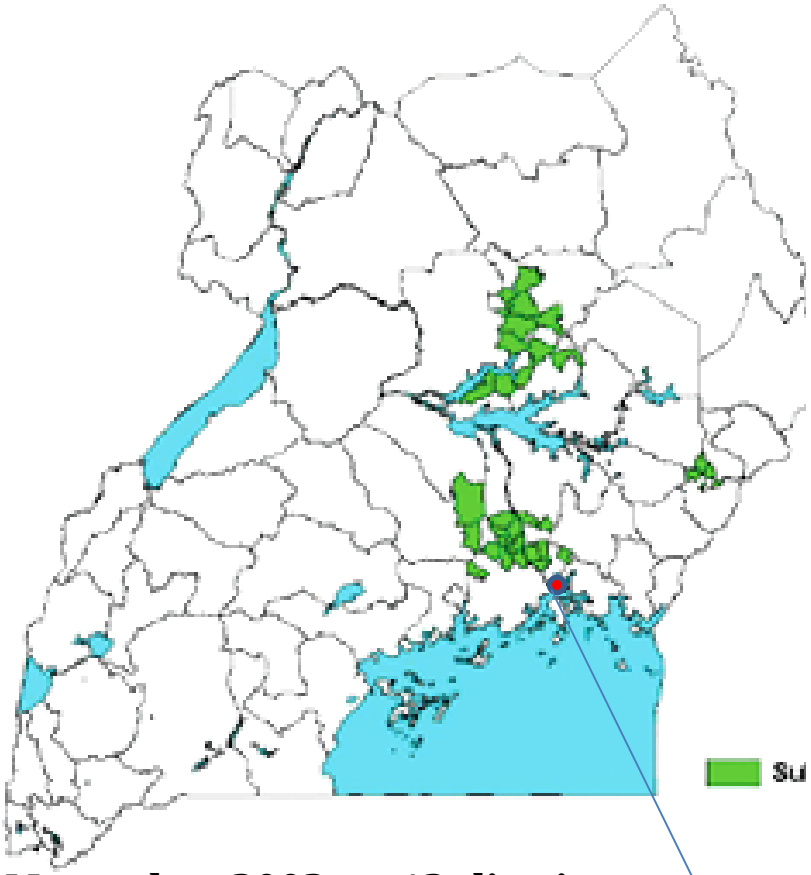
Banana Diseases

Banana Xanthomonas wilt (BXW)
Symptoms

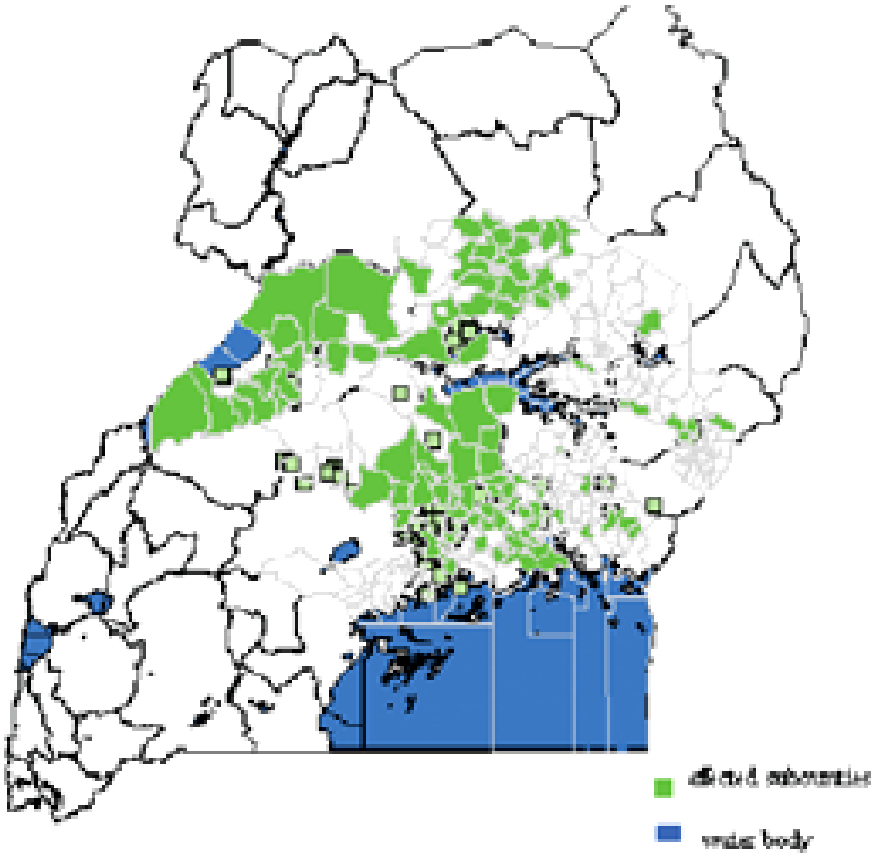
16. BXW –the leaf blade appears folded when on the plant
17. BXW – premature death of bracts is clearly shown
18. Fusarium wilt – the degree of yellowing varies yet with experience fungal wilt can be readily distinguished from BXW
19. BXW – yellow bacterial ooze
20. Fusarium – note darker staining inside the pseudostem
21. BXW – a less distinct example of bacterial wilt compared to other photographs but confirmed from fruit symptoms (Not shown)

Banana Xanthomonas wilt disease in Uganda

Pruning/cutting implement carry BBW inoculum



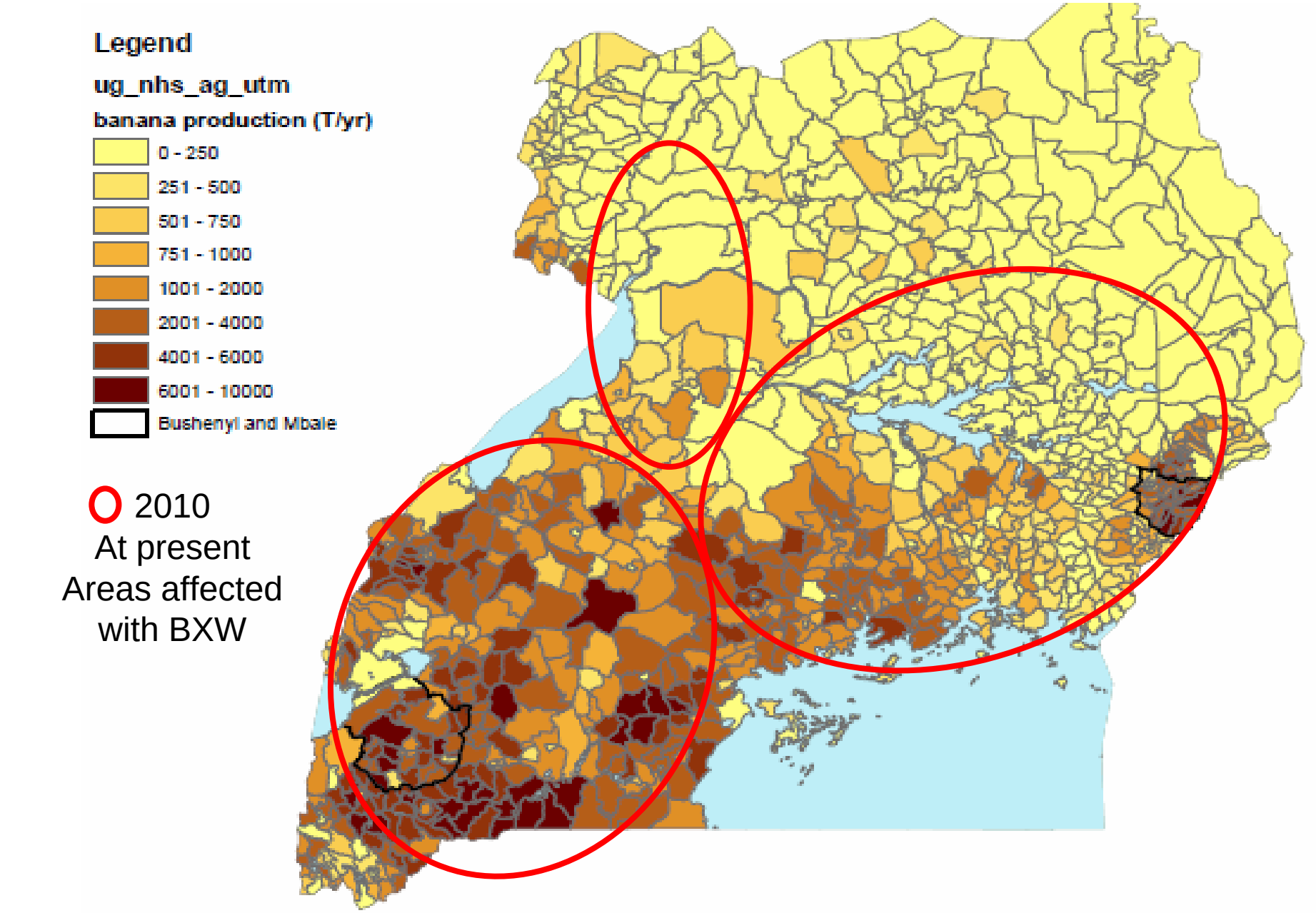
November 2003 — 12 districts



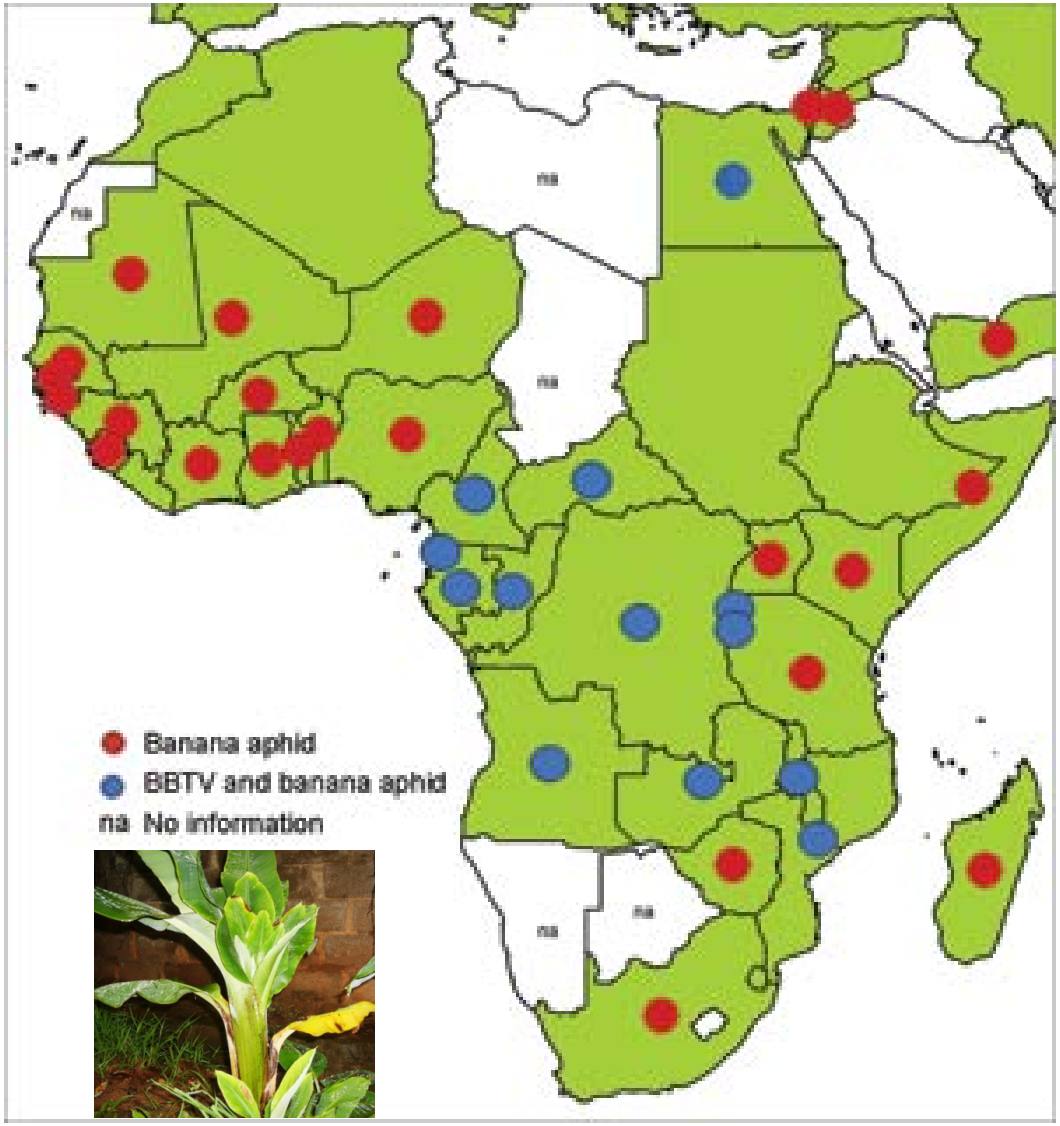
December 2004 — 26 districts

2001 -1st case of BXW in Mukono District

Figure 1. Distribution of BXW in Uganda:


Legend
ug_nhs_ag_utm
banana production (T/yr)
0 - 250
251 - 500
501 - 750
751 - 1000
1001 - 2000
2001 - 4000
4001 - 6000
6001 - 10000
Bushenyi and Mbale
2010
At present
Areas affected
with BXW

Banana Bunchy Top Disease (BBTD)



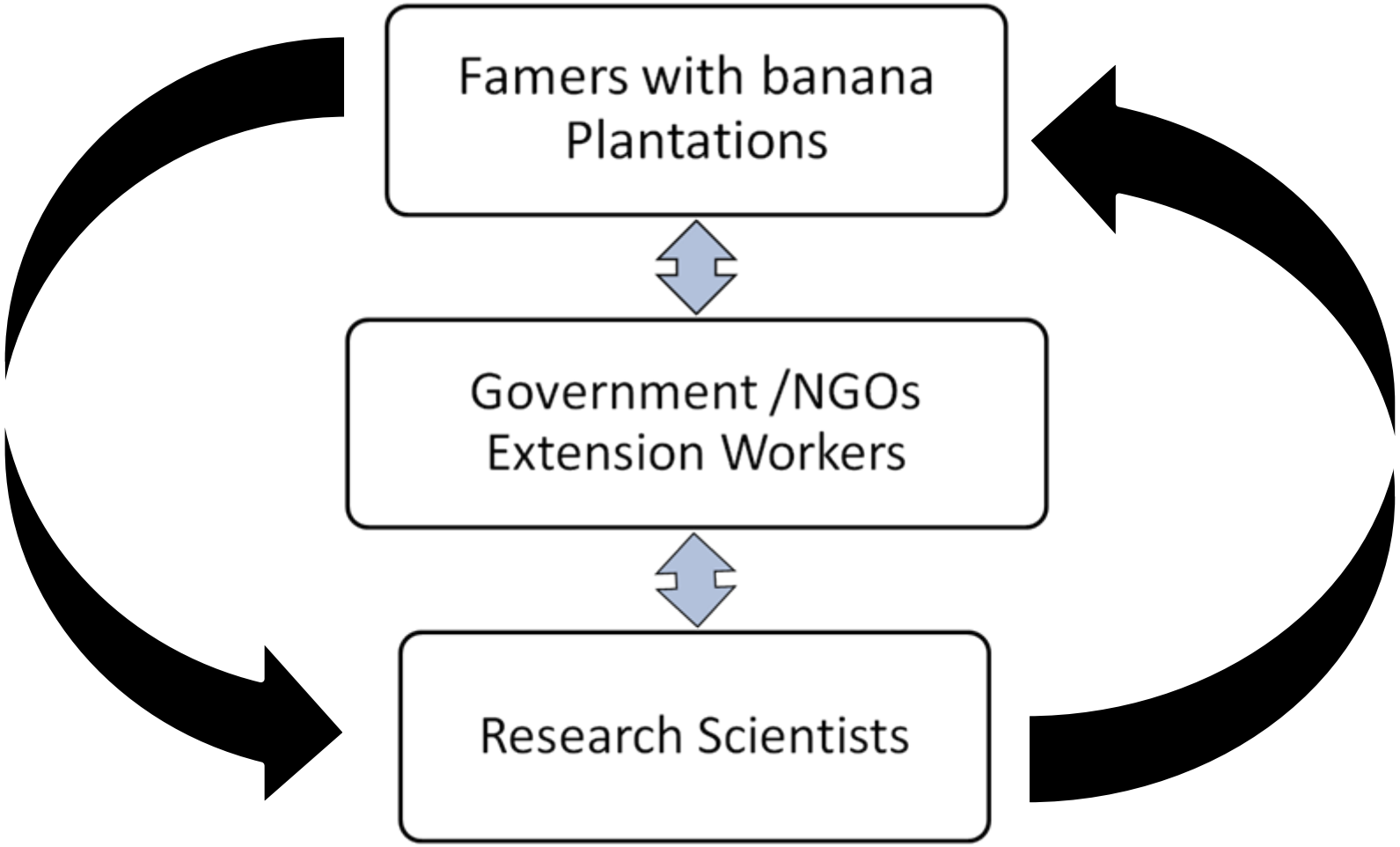
Banana Bunchy top Disease in Zambia

Before infection

After infection

Banana Bunchy top Disease in Burundi

Enhancing Banana disease surveillance



Enhance Information flow from Farmers to/from Scientists

Community Level Crop Disease Surveillance system (CLCDS).

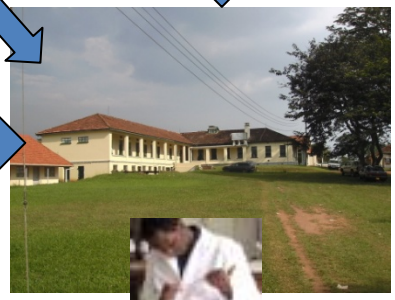
- To test the CLCDS concept, a multidisciplinary team consisting of professionals in the fields of plant pathology, agriculture-based data analysis, Geographic information systems, information and communication technology (ICT), and agriculture extension was assembled.

- The team developed a technological system and process to enable CKWs (Community Knowledge workers) to link to scientists to identify, map, monitor and control banana disease within their communities.

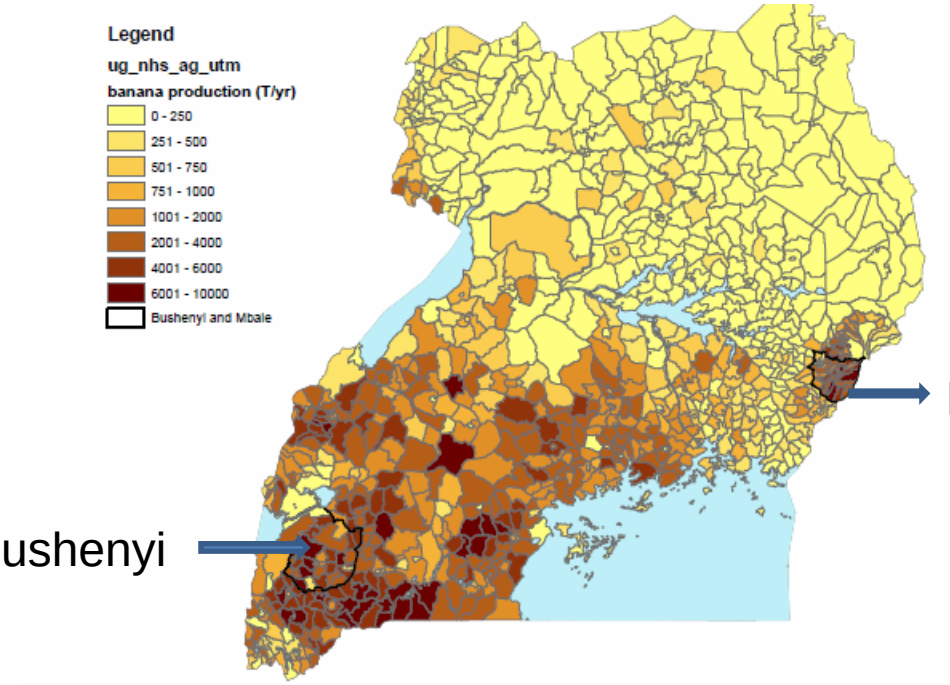
CKWs in Mbale

Internet Manager (Telecommunication (MTN) & (Applab)Soft ware developers

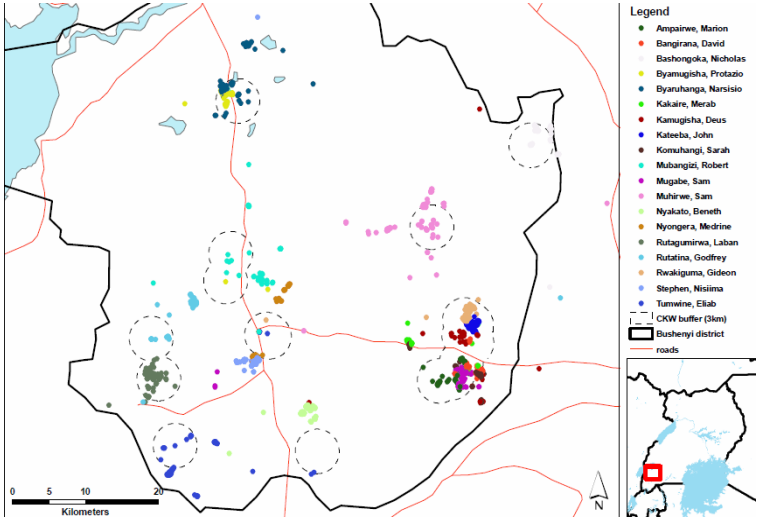
CKWs in Bushenyi



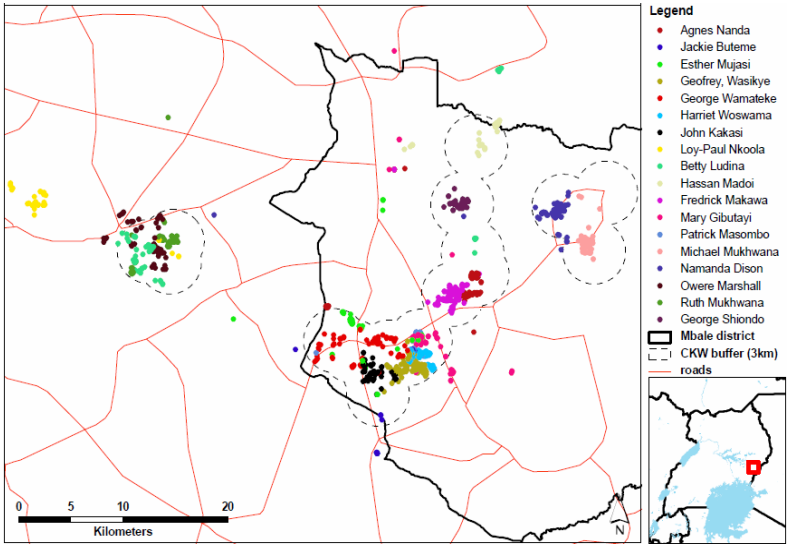
Research Scientists / stations



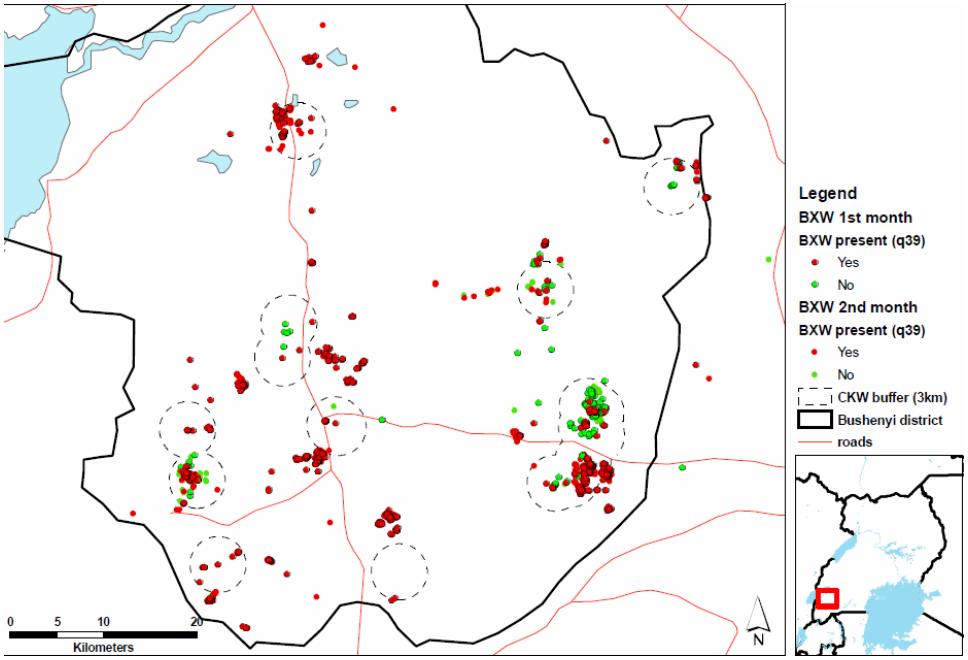
Surveys per CKW Bushenyi



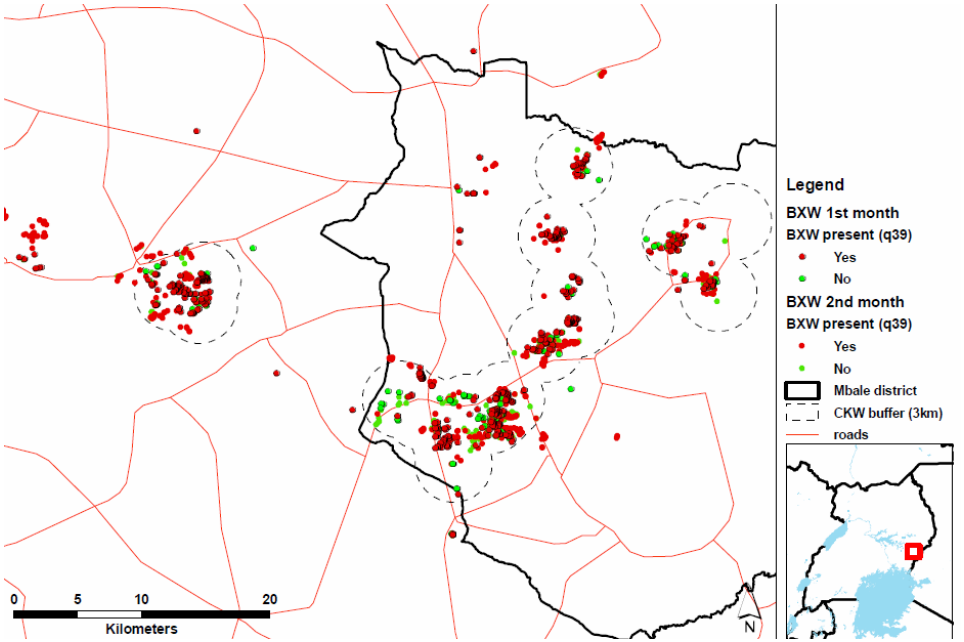
Surveys per CKW Mbale



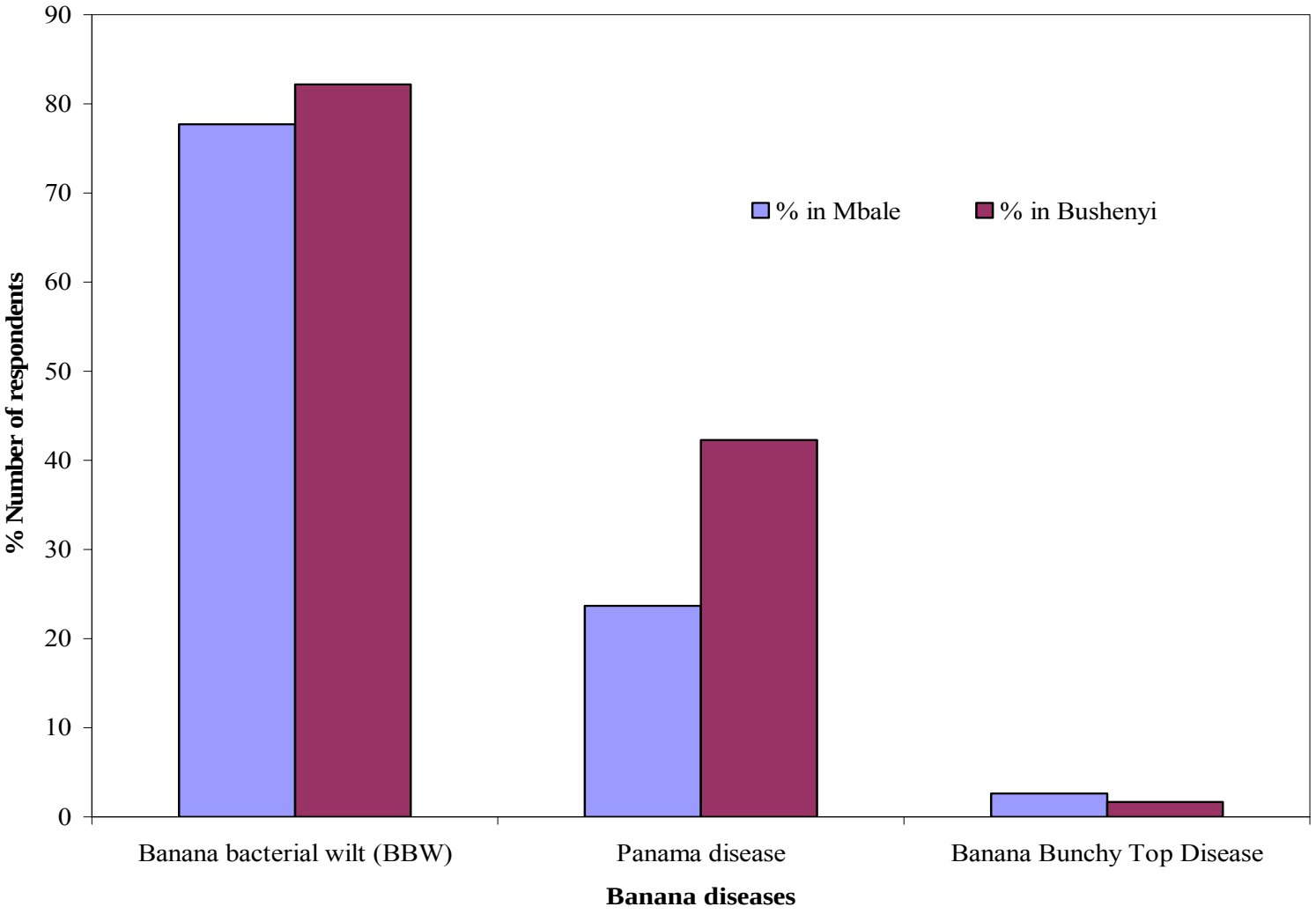
BXW incidence Bushenyi

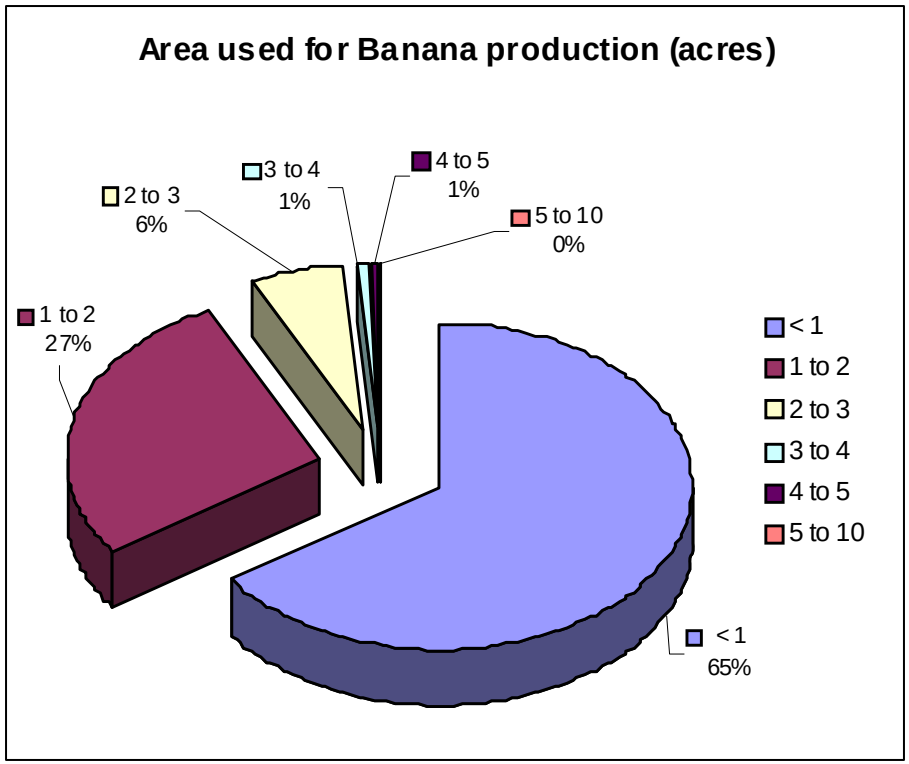


BXW incidence Mbale

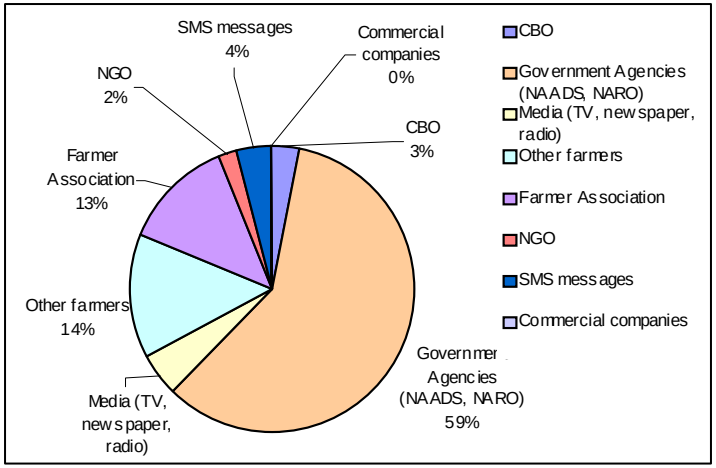


Farmer awareness of 3 targeted banana diseases





Source of information



Total farm area

Type of banana

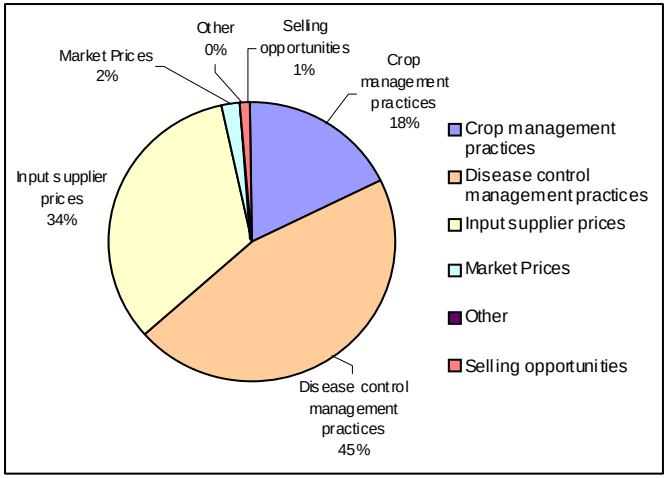
Other crops

Profit from banana

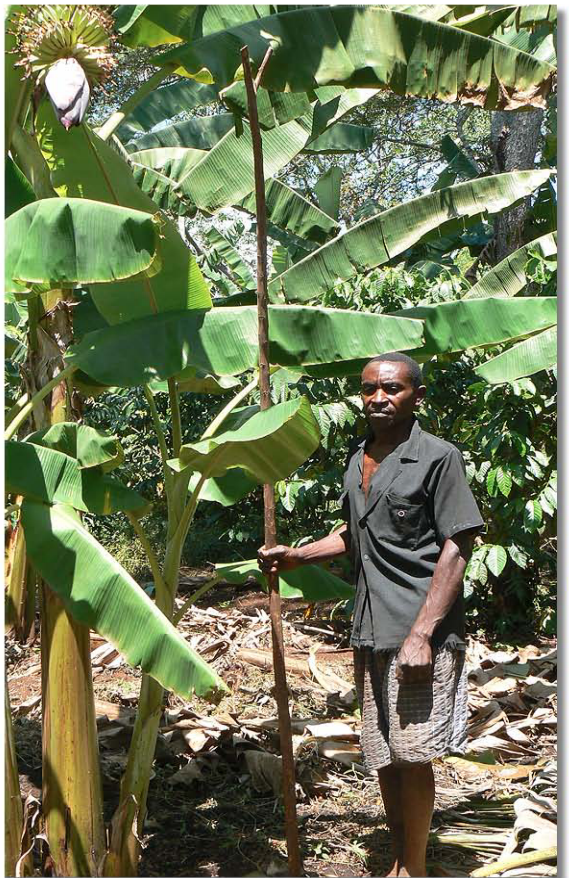
Market

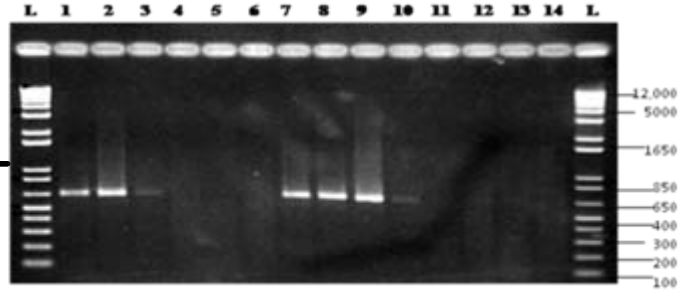
Planting material source

Information Requests



Banana Disease Surveillance Enhanced By Diagnostic Tools (Using Simple DNA Capture Kits)





DNA CAPTURE : So Far !!!!!!

There are several laboratory techniques using PCR that are available to diagnose plant pathogens based on the analysis of DNA/RNA.

Traditionally, tissues from diseased plants are used for this purpose.

However, on most occasions the time involved in dispatching samples from the field to the laboratory often results into their disintegration prior to arrival in the laboratory and loss of the integrity of pathogen DNA rendering detection problematic.

In addition, when the causal agent of diseased plant samples is suspected to be a bacterium or fungus; the first step on receipt in the laboratory is to recover living micro-organisms onto pure cultures prior to identification, which significantly delays the speed of diagnosis.

Also, sending diseased plant material imposes several bureaucratic and real quarantine hurdles in terms of the need for quarantine certificates, material transfer agreements, import and export permits, and avoiding introduction of a new disease variant.

However, sending nucleic acid samples poses no phytosanitary risk and thus obviates the need for such bureaucracy and time delay.

We are testing a range of methods to capture DNA from the field

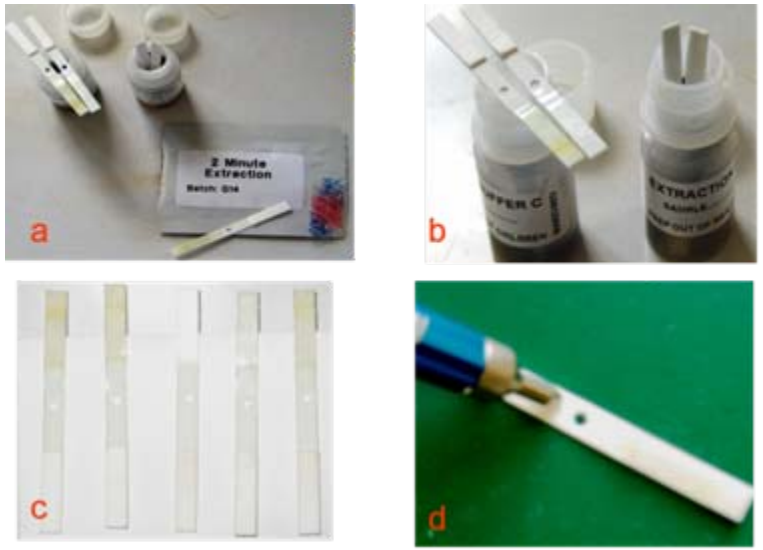
Three sample collection procedures, i.e., FTA cards, PhytoPASS and 2 minute extraction dip sticks were adopted.

There are no reports comparing different methods for collecting plant samples with respect to DNA yield and success in amplification under similar conditions, yet it is vital to know viability, efficiency and reliability of the sample collecting methods.

The availability of nucleotide sequences for *BBTV* and *XCM* made it possible for the development of PCR assays for detection and diagnosis.

FTA cards and reagents used

Two minute nucleic acid extraction dipstick



PhytoPASS and KAJI extraction buffer.

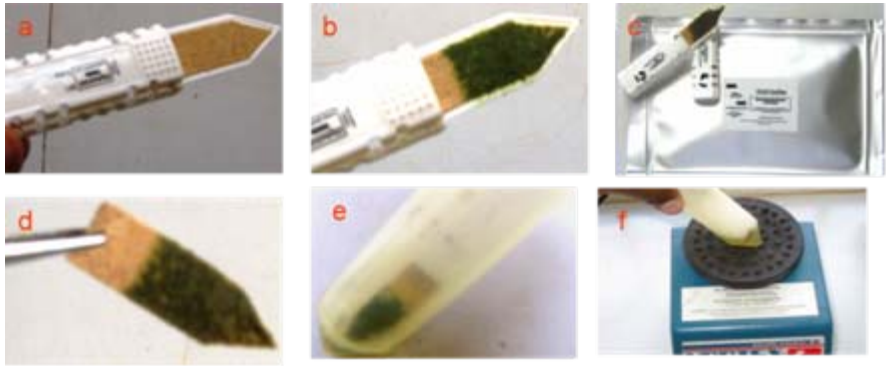
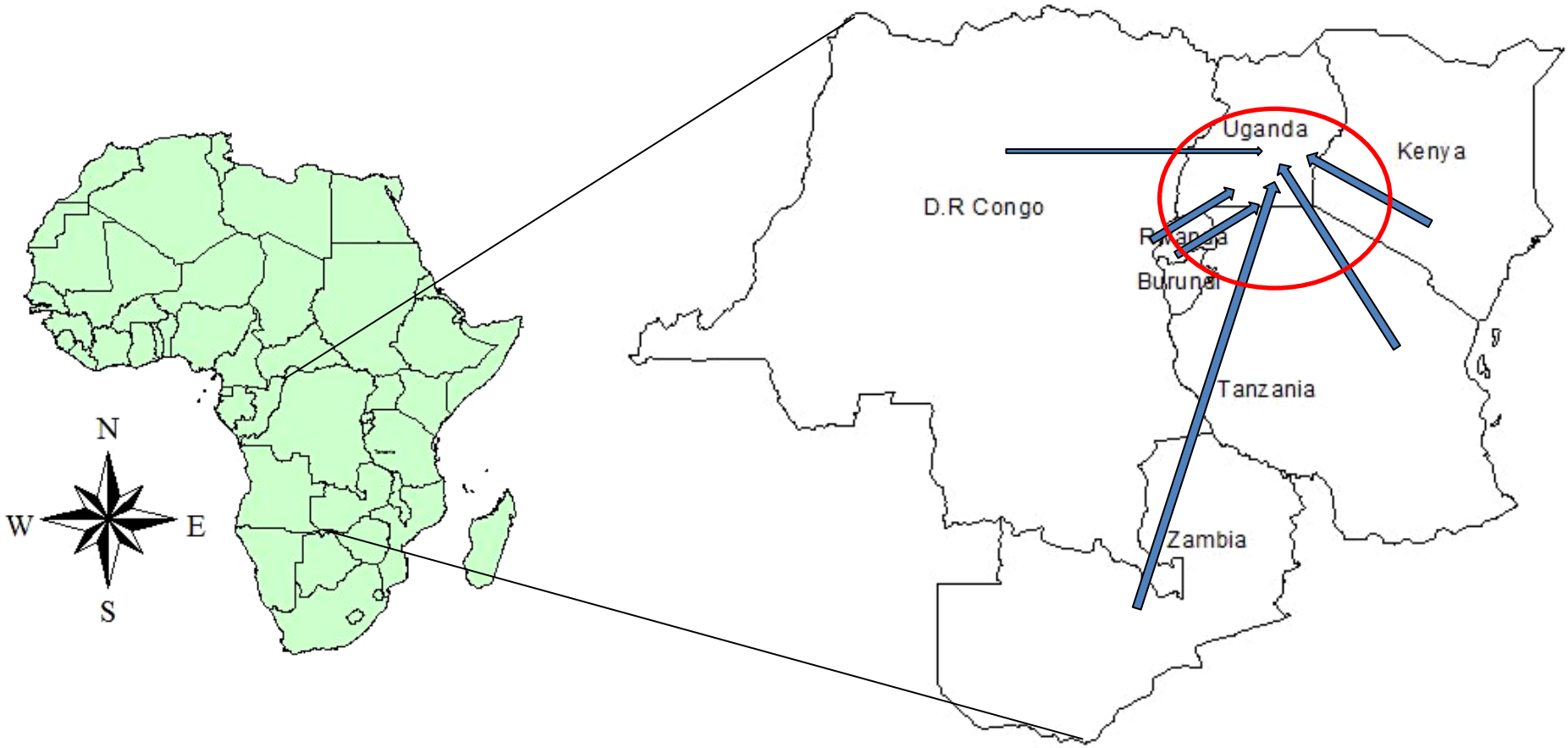
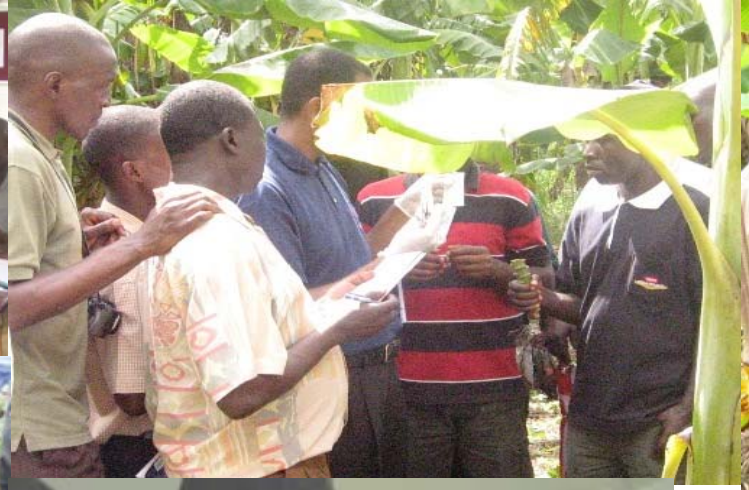


Fig 1. DNA sampling kit

Study area Targeting Banana Xanthomonas wilt and Banana Bunchy top Disease











Objectives

✍Optimise the DNA extraction protocols for FTA cards, PhytoPASS and 2 minute DNA dip stick using BBTV samples.

✍Optimise PCR conditions for detection of Xcm and BBTV genes.

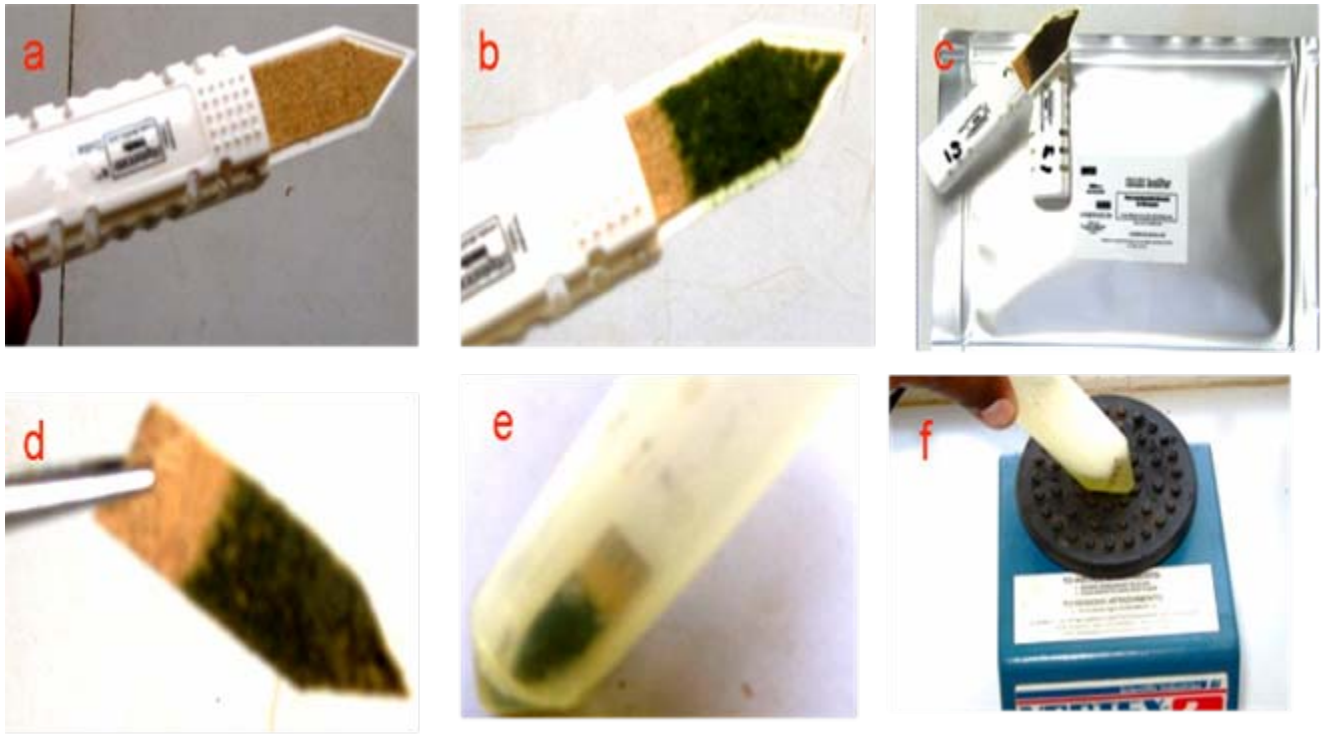
✍Test for the suitability, and efficacy of using these Kits to capture pathogen DNA

✍Test for storage capability of FTA cards, PhytoPASS and 2 minute DNA dip stick DNA capture kits for DNA capture of BXW and BBTV.

✍Test for repeatability of sample results from replicates between and within different DNA capture Kits for BXW and BBTV.

PHYTOPASS KIT

- PhytoPASS is a case containing a sampling strip carrying a sampling membrane.
- The sampling strip is rubbed on to the plant tissue, transversely to the fibres while avoiding over-saturating the sample membrane with tissues.
- They are easy to handle and do not need refrigeration.
- This kit has been employed in collection of Begomoviruses in cassava and BBTV.



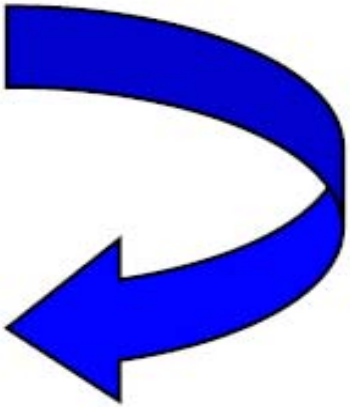
Whatman FTA Cards.

DNA extraction From FTA Card

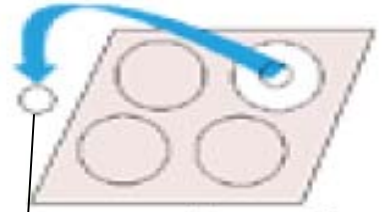
FTA card

1

Apply specimen and allow to dry completely



2

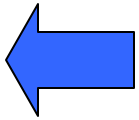


Punch samples

Area on the FTA card

3

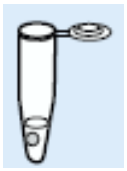
FTA Purification Reagent Washes
Place the disk in a PCR tube and wash three times with FTA Purification Reagent. Discard used reagent after each wash.



4

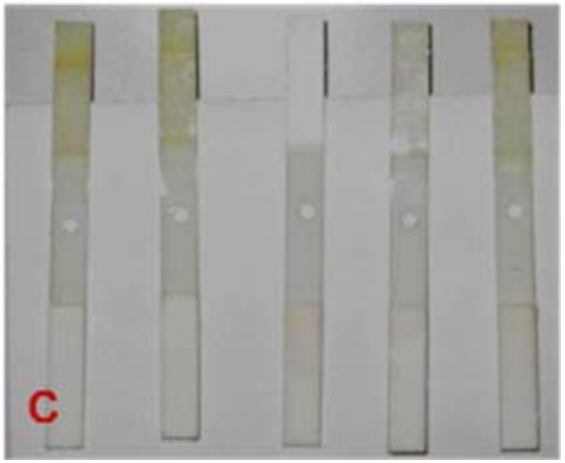
TE-1 Rinses
Wash twice with TE-1 buffer and discard used buffer after each wash. Dry disk in PCR tube.

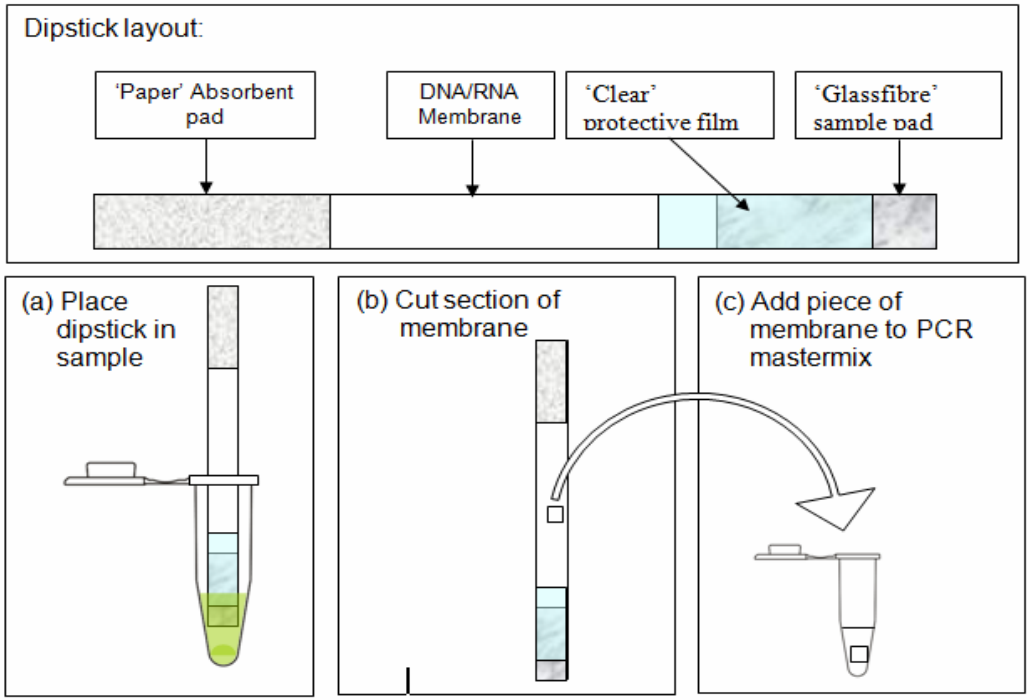
5



Direct to PCR
Add PCR master mix directly to the disk and amplify.

2 MINUTE EXTRACTION KIT.





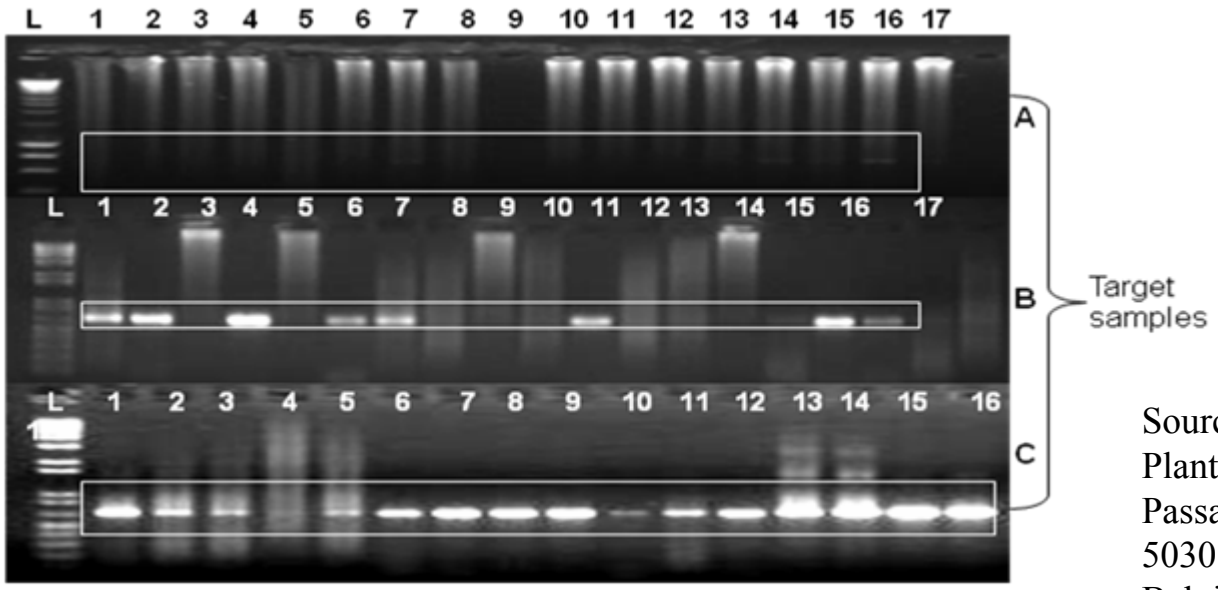
Results: Objective 1 Standardised DNA Extraction Protocols

PhytoPASS

: The sampling membrane is removed with tweezers and placed in a 10-ml glass tube containing 1 ml of cold ($^{-}4^{0}$C) KAJI extraction buffer (DNAlis sprl) and allowed to stand for 10 minute. Vortexing is performed to recover the plant tissue fragments in a suspension which is then kept on ice for about 5 minutes.

The obtained suspension constitutes the primary crude extract which is then diluted 100 times with distilled water before PCR analysis.

A=3ul
B=2ul
C=1ul



Source of kit :
Plant Pathology Unit
Passage des Déportés, 2
5030 Gembloux,
Belgium

Results: Standardised DNA Extraction Protocols

Method 1 (www.ftacard.com):

- Two discs are obtained from the imprinted FTA cards using a 2mm punch and placed in a PCR amplification tube.
- The discs are washed twice each time adding 50µl of FTA purification reagent and incubated for 5 minutes at room temperature and the washed solution discarded using a pipette.
- Discs are then washed once using 100 µl of TE^{-1} buffer or distilled water and once with 100 µl of absolute ethanol (to get rid of chlorophyll).
- The buffer and the ethanol are subsequently discarded using a pipette and the discs dried at 37^0C for 40 minutes in the oven.

Adjustment made to the protocol .

•Reduces numbers of washes to 2/3 (Several washes reduce DNA /RNA content)

•Lower wash volume (50 µl of FTA Purification regent to 30µl (100 µl to 5 µl of TE buffer)

•Allow - enough time for the FTA disc to Dry (Wet Disc inhibit PCR) 1-2hrs under room Temperature

FTA Card

Method 2 (Dellaporta *et al*., 1983, Rowhani *et al*., 2000):

- 1.0cm FTA card sample is soaked in 500µl of GEB buffer in a microfuge tube for 30 minutes at room temperature with occasional shaking.
- The extract is either used immediately or distributed into aliquots which were stored at −80 ^{o}C for subsequent use in PCR.
- 2 µl of the extract from above is mixed with 25 µl of GES buffer .
- The mixture is vortexed and heat denatured at 95^{0}C for 10 minutes in a water bath.
- The samples are then incubated on ice for 10 minutes. 1-3 µl of the contents is used in the PCR reaction as DNA template.

Adjustment made :

- Allow the FTA card to soak in the GEB buffer for 1 hour in case of Xcm and 30 minutes in case of BBTV
- Mix 50 µl of GEB extract with 250 µl of GES buffer and Incubate using a heating block set at 95 ^{o}C for 15 minutes

FTA card

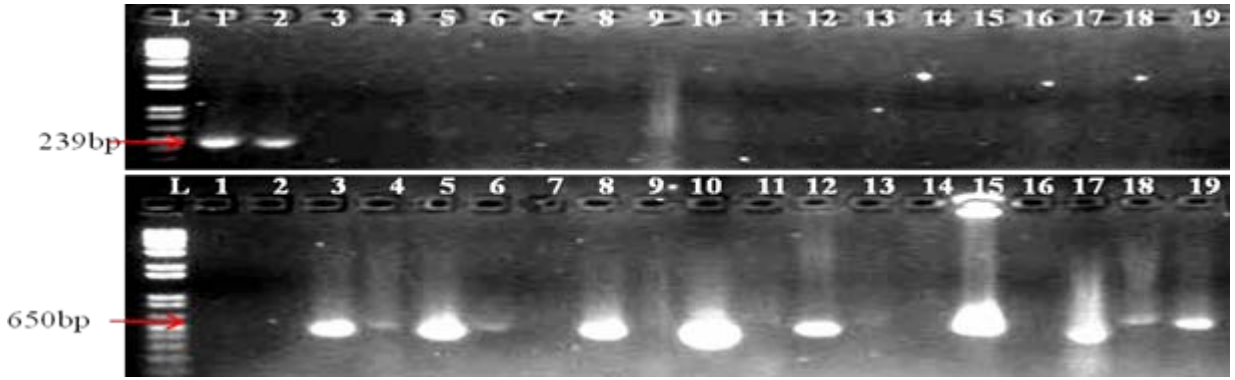


Figure 1 Amplification products from using FTA purification regent and TE buffer for DNA extraction from samples obtained using FTA cards. Gel (upper) indicates amplification product for BBTV gene (239 bp) detection using primer pair BBTV 1 /2 and gel (Lower) indicates amplification product for *Xcm* gene (650 bp) detection using primer pairs *Xcm* 38 F/R. Lane 1-19 are positive sample for BBTV (upper gel) and *Xcm* (Lower gel) respectively . Lane L is 100 bp ladders

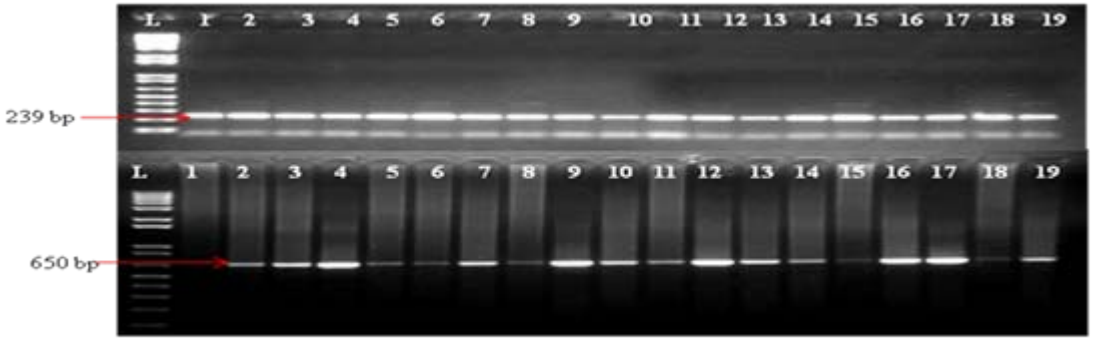
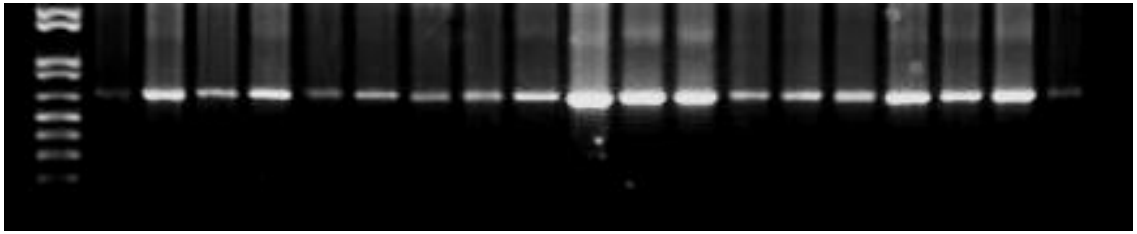
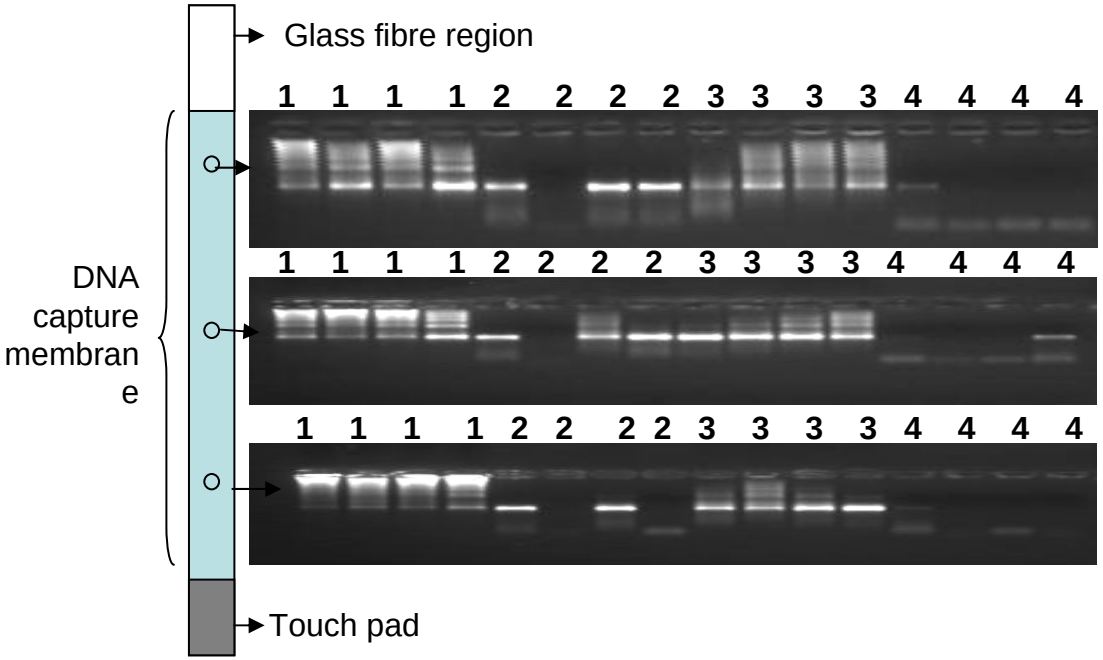


Figure 2 Amplification product from using GEB and GES buffer for DNA extraction from samples obtained using FTA cards. Gel (upper) indicates amplification product for BBTV gene (239 bp) detection using primer pair BBTV 1 /2 and gel (Lower) indicates amplification product for *Xcm* gene (650 bp) detection using primer pairs *Xcm* 38 F/R. Lane 1-19 are positive sample for BBTV (upper gel) and *Xcm* (Lower gel) respectively . Lane L is 100 bp ladders

2 minute DNA extraction Dipstick



Amplification product from using a single 2mm disc from a DNA membrane of a Two minute DNA dipsticks. Product indicates amplification product for BBTV gene (239 bp) detection using primer pair BBTV 1 /2 Lane L is 100 bp ladders



Amplification of *Xcm* DNA obtained at varying distance along a dipstick as well as among replicate sample dipsticks. 1 -4 are different samples with their respective replicate (4 in number)

A single 2 mm disc is adequate for PCR amplification and from a single dipstick you can cut out about 10-12 separate discs for separate reactions

Optimal DNA template for detection of *Xcm* and BBTV

The optimal DNA template for detection of both pathogens

- ✓Two minute DNA dipstick kits -a single 2 mm disc of DNA membrane,
- ✓FTA card - 2 (2mm) discs for FTA purification reagent or
  - <1-3ul of FTA card extract using GES and BEB buffer
- ✓PhytoPASS - <1-3ul of the 100x dilution extract of the PhytoPASS membrane.

Optimal PCR conditions for detection of *Xcm* and BBTV

Amplification with these respective quantities of the template yielded the expected PCR product of 650 bp (Figure A) at an optimal annealing temperature of 60^0C for *Xcm* at 40 cycles and PCR product of 239 bp (Figure below) over a range of annealing temperatures (50^0C -60^0C) for BBTV at 35 cycles . The optimal annealing temperature for BBTV was 53^0C since it gave the most appropriate band

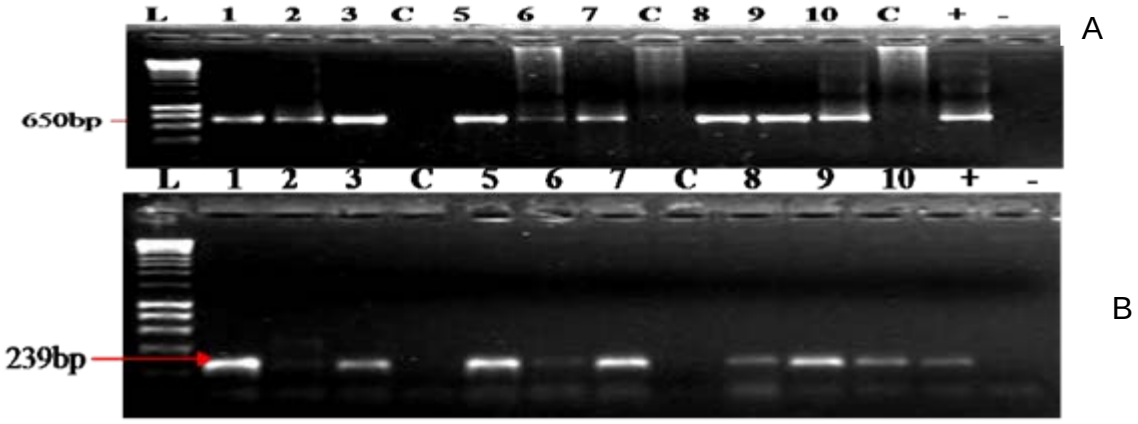


Figure . PCR detection of *Xcm* and BBTV from infected plants. (A) Ethidium bromide-stained amplification products (650bp) obtained with primers *XCM* /38 F and *XCM*/38 R from DNA obtained using three DNA capture kits, 1-3(2 minute DNA extraction dip sticks), 5-7(PhytoPASS kit samples) , 8-10(FTA card). Lane + and C are positive and negative control respectively. L is a molecular size marker. B Ethidium bromide-stained amplification products (239 bp), obtained with primers BBTV 1 and BBTV 2 from DNA obtained using three DNA capture kits, 1-3(2 minute DNA extraction dip sticks), 5-7(PhytoPASS kit samples), 8-10(FTA card using purification reagent). Lane + and C are positive and negative control respectively. L is a molecular size marker.

Repeatability of sample results from replicates between and within different DNA captures Kits for *Xcm* and BBTV.

DNA capture Kits	Two minute DNA dipsticks				PhytoPASS				Whatman FTA card			
Replication per Kit	Rep 1	Rep 2	Rep 3	Controls	Rep 1	Rep 2	Rep 3	Control	Rep 1	Rep 2	Rep 3	Control
Expected Amplification	45	45	45	45	45	45	45	45	45	45	45	45
Actual Amplification	44	40	42	8	35	40	43	10	43	40	43	6
BBTV % Amplification	97.8	88.9	93.3	17.8	77.8	88.9	95.6	22.2	95.6	88.9	95.6	13.3

DNA capture Kits	Two minute DNA dipsticks				PhytoPASS				Whatman FTA card			
Replication per Kit	Rep 1	Rep 2	Rep 3	Rep 4©	Rep 1	Rep 2	Rep 3	Rep 4 ©	Rep 1	Rep 2	Rep 3	Rep 4 ©
Expected Amplification	28	28	28	28	28	28	28	28	28	28	28	28
Actual Amplification	22	22	19	19	20	16	18	13	28	23	24	23
Xcm % Amplification	78.6	78.6	67.9	67.9	71.4	57.1	64.3	46.4	100.0	82.1	85.7	82.1

Repeatability of Amplification

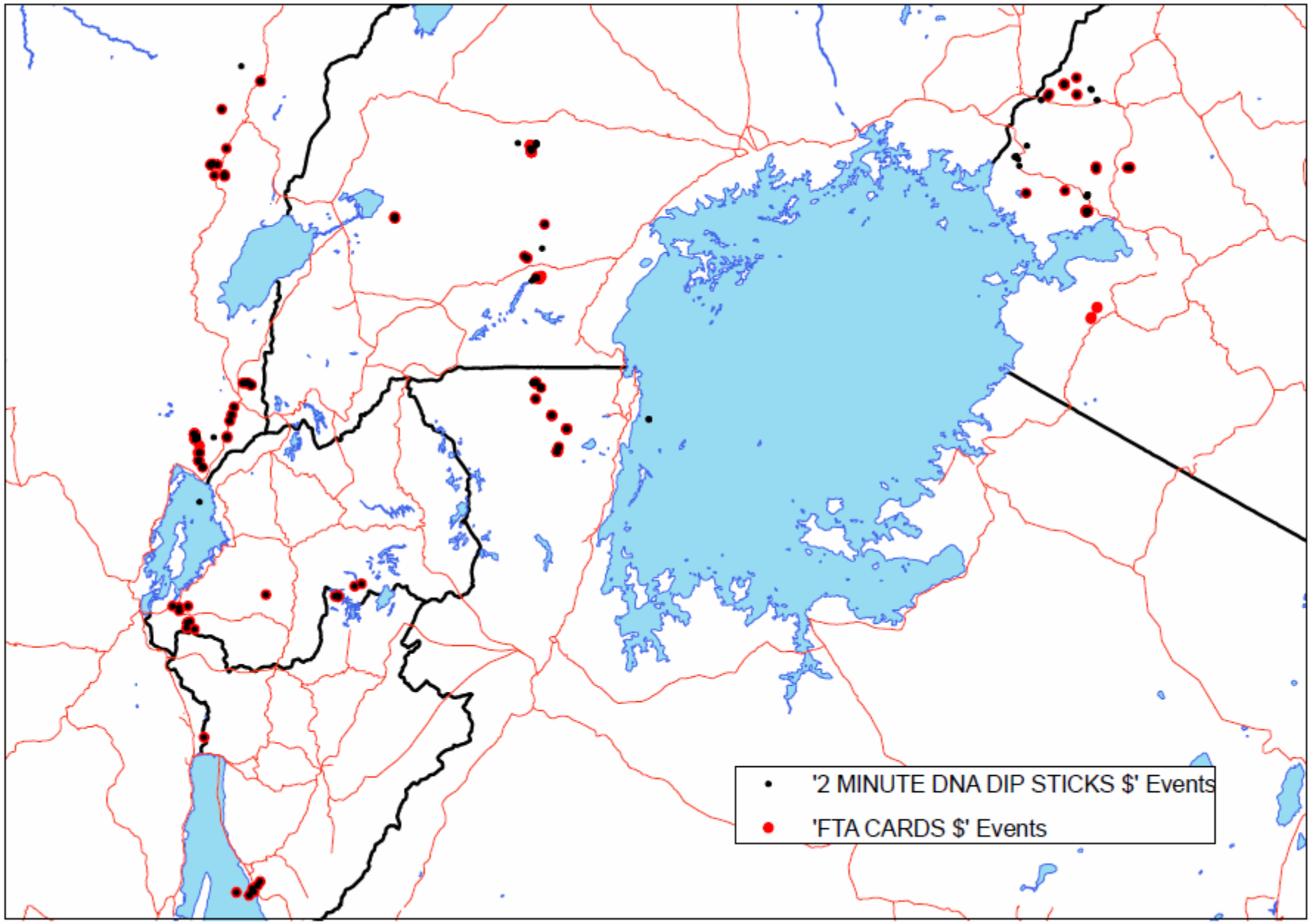
PhytoPASS (78% - 96%)
2 minute DNA dipsticks (89% -96%)
FTA card (89-96%) Based on GEB and GES buffer
however
Based on FTA Purification reagent and TE buffer
FTA Card (Average 57.9%)

Deduction :Based on BBTV detection data

Confirmed cases of *Xanthomonas campestris pv musacearum* presences in the different countries sampled using 2 minute DNA dipstick and FTA card DNA capture kits

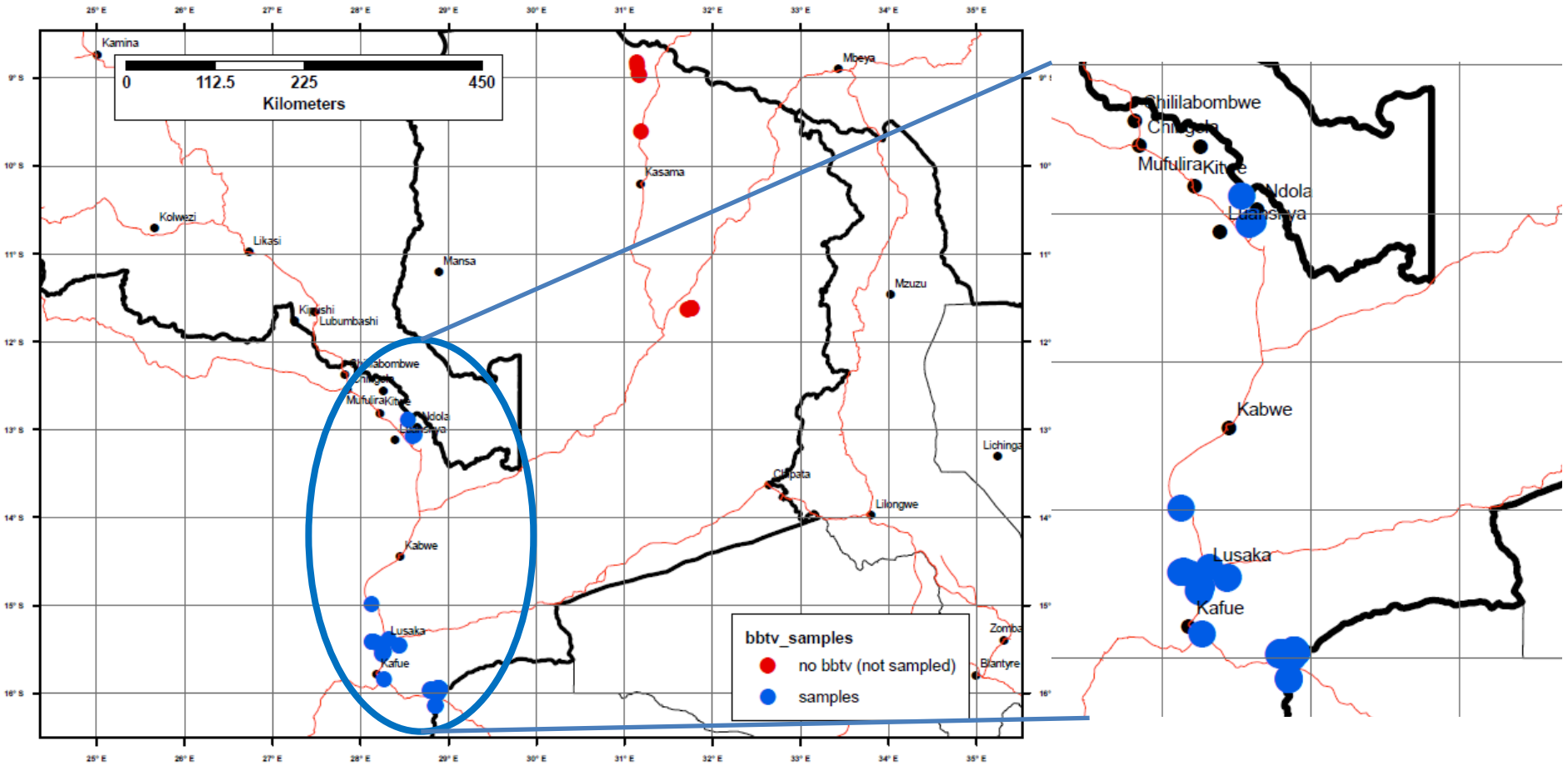
Submitted samples included symptomatic, asymptomatic and healthy samples.

Country	2 minute DNA dipsticks			FTA card (Based on GEB and GES buffer)		
	Diseased samples	Suspected Samples	Healthy Samples	Diseased samples	Suspected Samples	Healthy Samples
D.R Congo	25	30	5	25	0	5
Confirmed Xcm	18	0	0	19	0	2
%	**72**	0	0	**76**	0	40
Kenya	23	15	5	16	13	5
Confirmed Xcm	9	5	2	10	4	1
%	**39**	33	40	**63**	31	20
Rwanda	30	0	30	27	0	3
Confirmed Xcm	25	0	15	21	0	1
%	**83**	0	50	**78**	0	33
Burundi	2	23	5	3	22	2
Confirmed Xcm	1	9	4	2	2	2
%	**50**	39	80	**67**	9	100
Tanzania	20	30	10	26	0	4
Confirmed Xcm	17	12	1	16	0	1
%	**85**	40	10	62	0	25
Uganda	20	13	8	18	4	8
Confirmed Xcm	14	8	4	14	1	1
%	70	62	50	78	25	13



Areas sampled in Uganda ,Kenya ,Tanzania, D.R Congo , Rwanda and Burundi

Areas sampled in Zambia for BBTV



Storage duration and viability for FTA cards, PhytoPASS and 2 minute DNA dip stick kits

Results represented above are the results of the lab analysis carried on to the samples with the longest duration of storage for

- **2minute dipstick - 153 days (> 5 months under storage)**
- **FTA card - 185 days (> 6 month under storage)**
- **PhytoPASS -120 days (> 3 month under storage)**

The result indicate that these DNA capture kits can hold the integrity of DNA for a longer duration under room temperature conditions before any analysis carried out .

	Sample collection date	Data of Laboratory Analysis		
Country	Both DNA sampling Kits	2 minute DNA dipsticks	FTA card	Duration under storage
D.R Congo	08th /02/2010	26th/05/2010	28/06/2101	107-140 days
Kenya	17th/02/2010	27th/05/2010	4 /07/2010	99-105days
Rwanda	24th /3/2010	02nd/06/2010	1/07/2010	70-99days
Burundi	30/12/2000	02nd/06/2010	4/07/2010	153-185days
Tanzania	02nd /02/2010	26th/05/2010	28/06/2010	113-145days
Uganda	10th /03/2010	02nd/06/2010	1/07/2010	84-113 days

Table : Duration under storage from the time the sample were collected from the field to laboratory analysis

Testing the sensitivity of 2 minute DNA dipstick and FTA card based on Conventional PCR

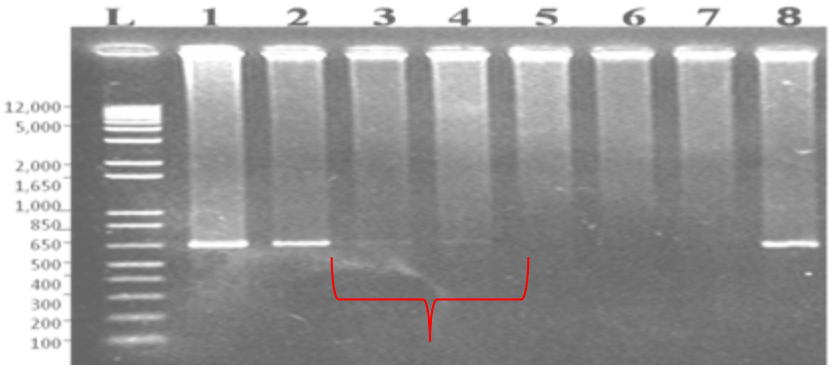


Figure Detection of *Xanthomonas campestris pv musacearum* (*XCM*) DNA using serial dilution of varying *Xcm* concentration input on FTA card matrix, then extraction and PCR performed using primer specific for production of a 650 bp band characteristic of *XCM* .Lane 1 -4 showed detectable amplification with very weak band with lane 3 and 4 and undetectable bands with lane 6 -7. Lane 1- 10, 2-$10^{-1,}$ 3-$10^{-2,}$ 4-$10^{-3,}$ 5-10^{-4} 6-$10^{-5,\ 7}$-10^{-6} and C as the positive control *XCM* plasmid DNA. L is 1 kb plus DNA Ladder

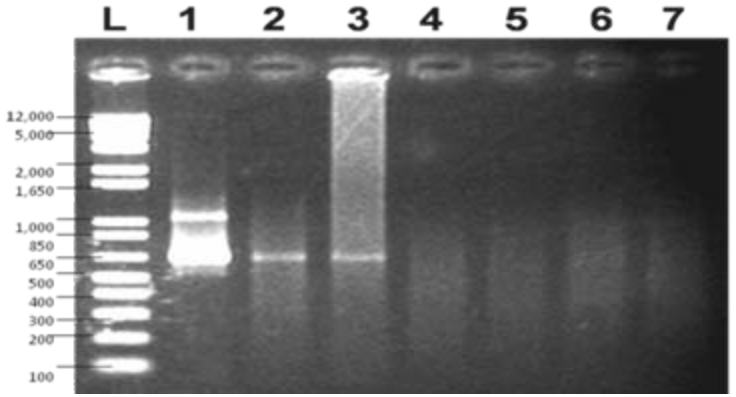
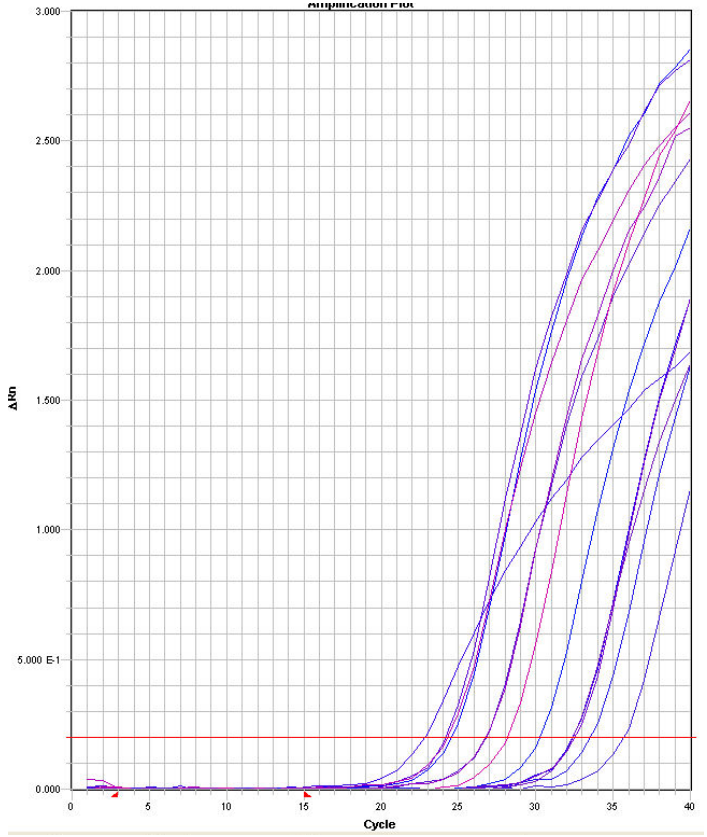


Figure . *Xanthomonas campestris pv musacearum* (*Xcm*) detection using varying *Xcm* concentration input on 2 minute DNA test dipsticks .Lane 1 -3 showed detectable amplification with reducing intensity of band with lane 2 and 3 . Lane 1- 10, 2-10^{-1}, 3-10^{-2}, 4-10^{-3}, 5-10^{-4} 6-10^{-5} and 7-10^{-6}. L is 1 kb plus DNA Ladder

FTA card : Minimal concentration detectable 10^{-3} dilution factor/0.011(absorbance/spectrophotometer reading at 600nm) or $55*10^2$ cfu/ml of *Xcm* solution

Two Minute DNA dipsticks 10^{-2} dilution factor) or $88*10^2$ cfu/ml of *xcm* solution .

Testing the sensitivity of 2 minute DNA dipstick and FTA card based on Taqman PCR



Xcm concentration	Dilution factor	Spectrophotometer reading at 600nm*	Colony forming units (cfu /ml)	Average Ct values	Average ΛRXN
1	10	2.333	> 141* 10^2	23	2.8
2	10^{-1}	0.178	141-116*10^2	24	2.6
3	10^{-2}	0.125	80-96*10^2	27	2.4
4	10^{-3}	0.011	57-53*10^2	26	2.1
5	10^{-4}	0.009	23-37*10^2	30	1.8
6	10^{-5}	0.006	21-20*10^2	32	1.6
7	10^{-6}	0.001	<20*10^2	35	1.1

Economics of using DNA capture kits

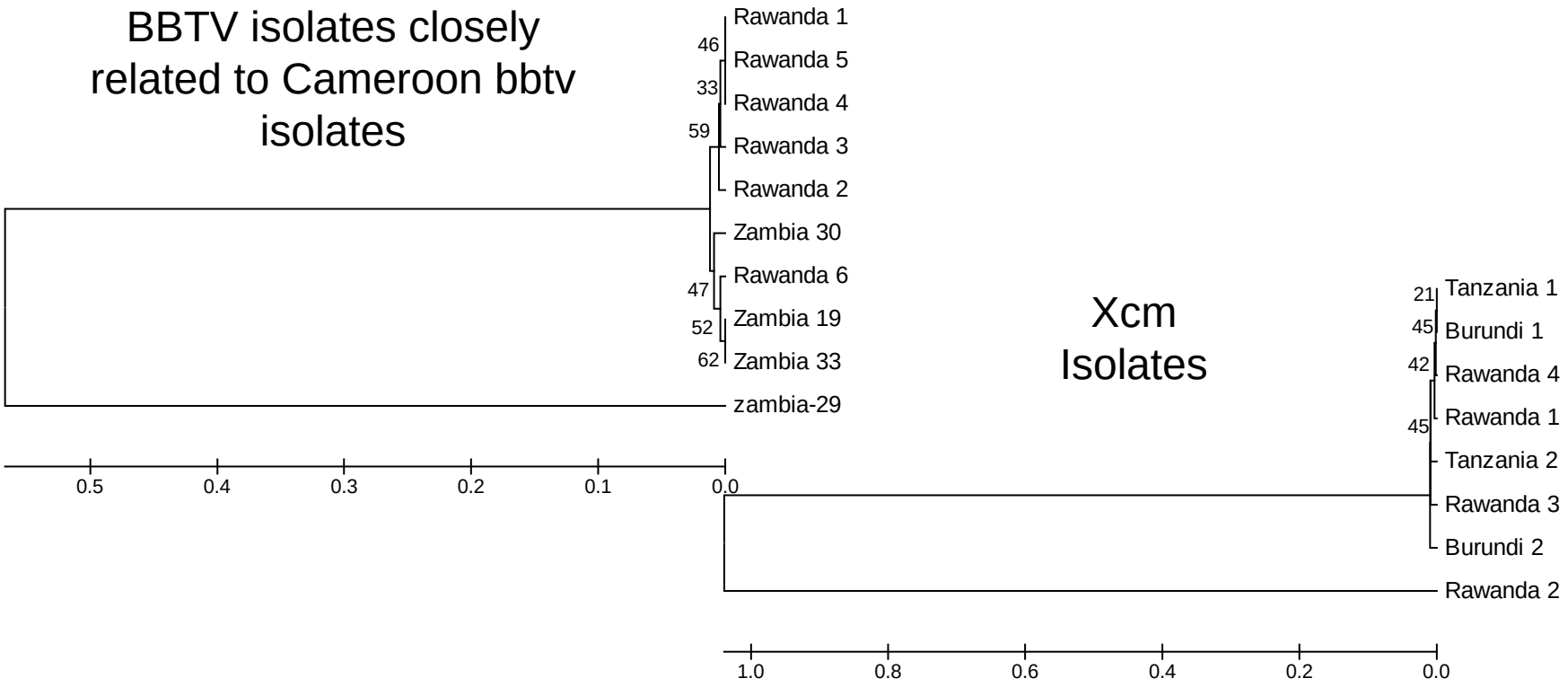
Parameter	2 minute DNA dipsticks	PhytoPASS Kit	Whatman FT A Card
Unit cost	3.78£	2.72£	3.09£
Ease of use in the field •1-Much easier •2 –Easier •3-With Difficulty	2 (involves shaking the extraction bottle for a 30-60 seconds)	1 (involves rubbing the PhytoPASS membrane transversely across a diseased tissue)	3 (Pressing the sampled onto the FTA card matrix)
Ease of DNA extraction •1-Much easier •2 –Easier •3-With Difficulty	1 Performed during Sampling Takes 2 minutes	2 Only a single Buffer is used (KAJI buffer) Takes 5 minutes	3 With GES and GEB buffer takes 30 minute while with Purification reagent and TE buffer , takes 45 minutes-1 hour
Ease of PCR diagnosis •1-Much easier •2 –Easier •3-With Difficulty	1 (Good Amplification)	2 (Good amplification, but requires further dilution of extract	3 Good amplification but requires further dilution of extract obtained using GES and GEB buffer. Uncertain (chances of losing DNA from the FTA card discs are high when washed with Purification reagent and TE buffer
Degree of Repeatability of Amplification	68-97%	57-96%	82-100%
Storage capability	DNA stable at 3 month minimum (tested duration)		
Duration taken for • DNA extraction	N/A	15-30 minute	1hr-45 minutes using GEB and GES buffers or Purification reagent / TE buffer
• PCR	1-2 hrs	1-2 hrs	1-2 hrs
• Gel Electrophoresis and visualisation	1 hr	1 hr	1 hr
Time Taken for diagnosis	2-3hr	2-3½hrs	3-4hrs

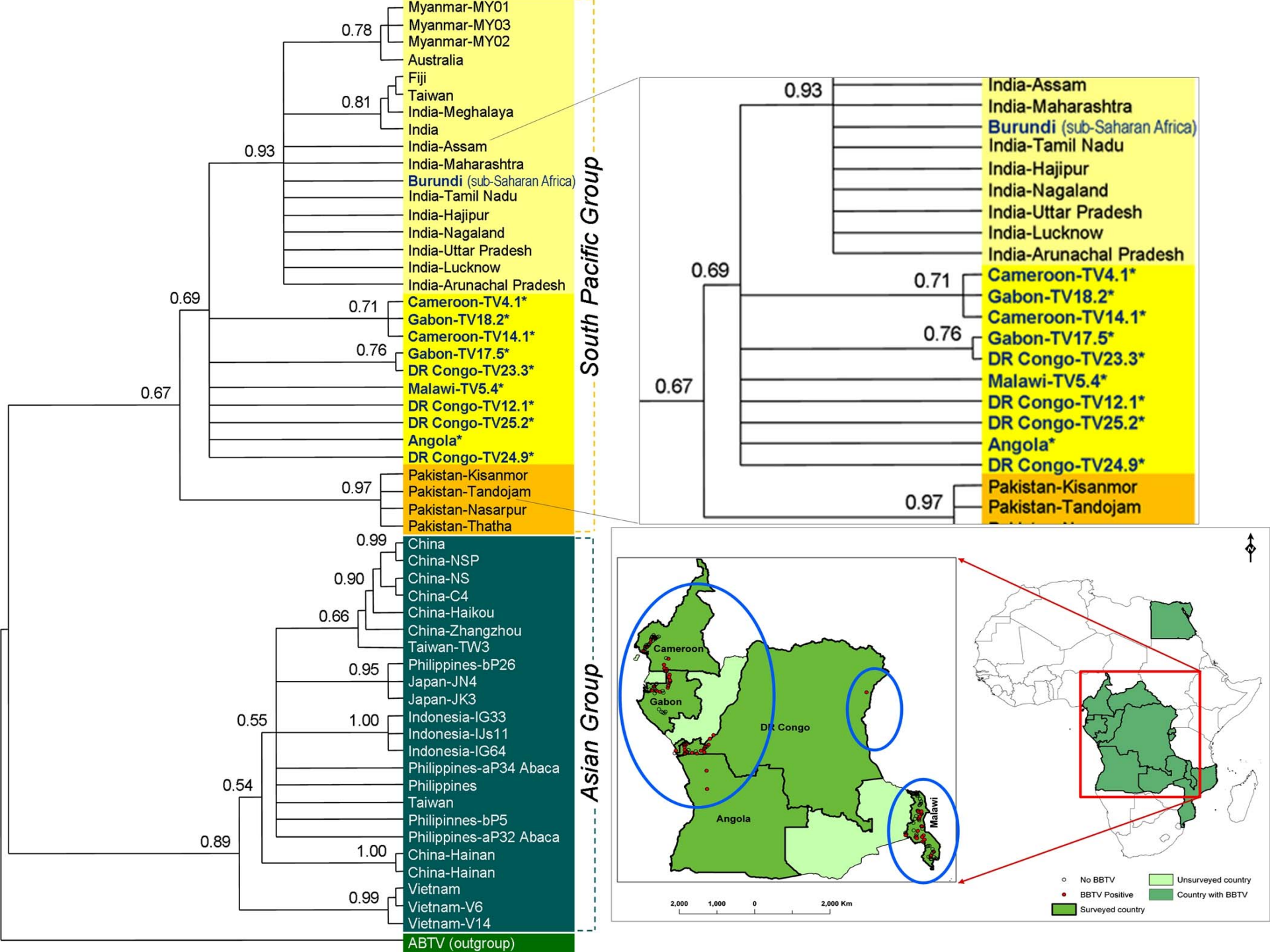
Work on-going

Variance Studies on

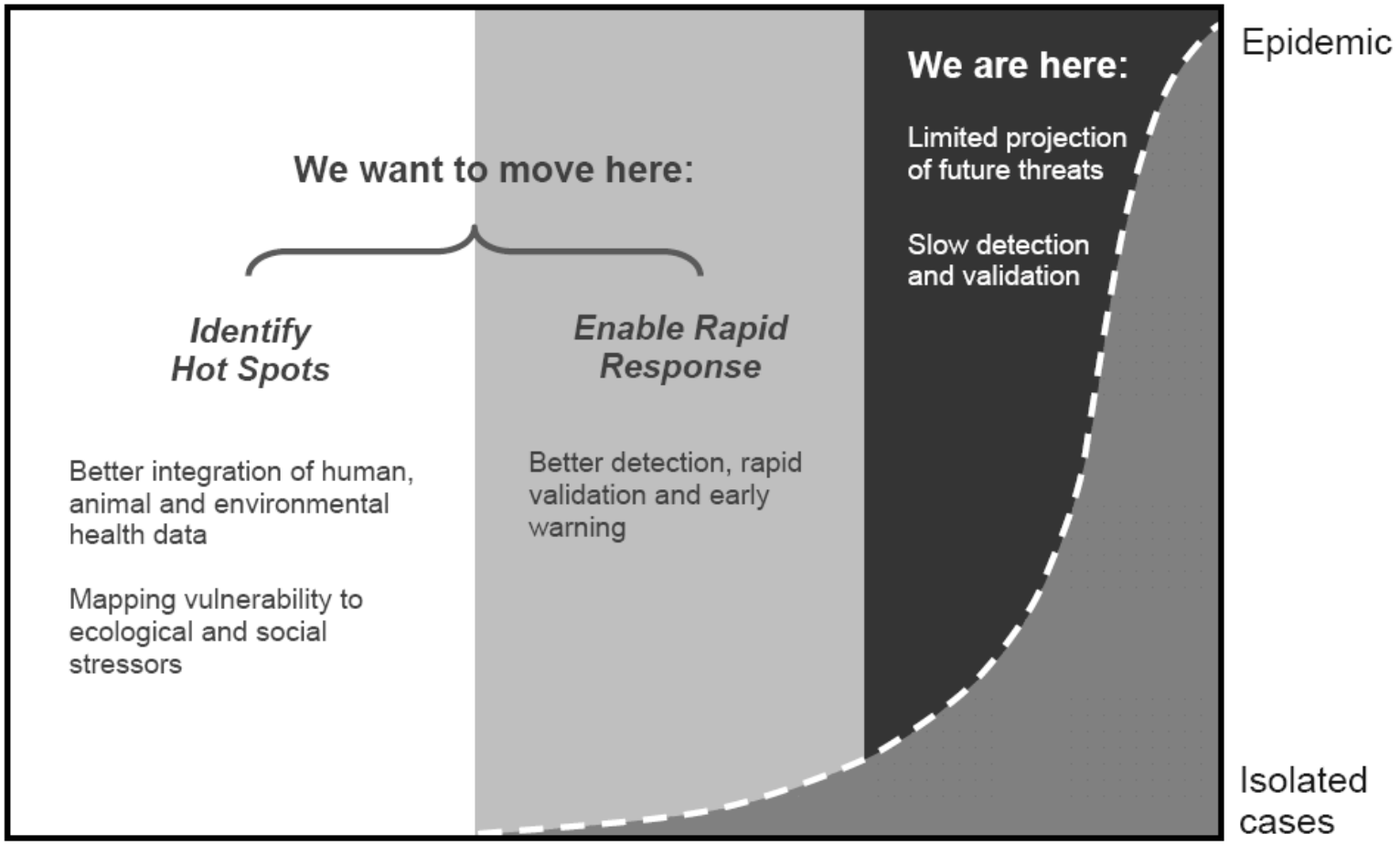
-Xcm Isolates from (Uganda ,Kenya ,Tanzania , Burundi , Rwanda and D.R Congo

-BBTV Isolates from Rwanda ,Zambia , Burundi (Based on Sequencing and alignment of Sequences)




Myanmar-MY01
Myanmar-MY03
Myanmar-MY02
Australia
Fiji
Taiwan
India-Meghalaya
India
India-Assam
India-Maharashtra
Burundi (sub-Saharan Africa)
India-Tamil Nadu
India-Hajipur
India-Nagaland
India-Uttar Pradesh
India-Lucknow
India-Arunachal Pradesh
Cameroon-TV4.1*
Gabon-TV18.2*
Cameroon-TV14.1*
Gabon-TV17.5*
DR Congo-TV23.3*
Malawi-TV5.4*
DR Congo-TV12.1*
DR Congo-TV25.2*
Angola*
DR Congo-TV24.9*
Pakistan-Kisanmor
Pakistan-Tandojam
Pakistan-Nasarpur
Pakistan-Thatha
China
China-NSP
China-NS
China-C4
China-Haikou
China-Zhangzhou
Taiwan-TW3
Philippines-bP26
Japan-JN4
Japan-JK3
Indonesia-IG33
Indonesia-IJs11
Indonesia-IG64
Philippines-aP34 Abaca
Philippines
Taiwan
Philipinnes-bP5
Philippines-aP32 Abaca
China-Hainan
China-Hainan
Vietnam
Vietnam-V6
Vietnam-V14
ABTV (outgroup)
South Pacific Group
Asian Group
0.78
0.81
0.93
0.69
0.71
0.76
0.67
0.97
0.99
0.90
0.66
0.95
0.55
1.00
0.54
0.89
1.00
0.99
Cameroon
Gabon
DR Congo
Angola
Malawi
2,000 1,000 0 2,000 Km
No BBTV
BBTV Positive
Surveyed country
Unsurveyed country
Country with BBTV

Conclusion – Enhancing Banana disease Surveillances



Google: Predict and Prevent

fera

The Food and Environment Research Agency